BIOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF A GROUP-SPECIFIC ANTIGEN FROM *STREPTOCOCCUS MUTANS* AHT

By

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A membrane-associated glycerol teichoic acid antigen has been isolated from *S. mutans* AHT and a similar antigen has been demonstrated to be present in each of the other Bratthall group *a* organisms studied. Trichloroacetic acid-extracted material was resolved into two phosphorus-containing antigenic fractions (B and C) by agarose chromatography. Fraction B was preliminarily identified as a phospholipid moiety with a glycerol to phosphorus ratio of 2:1 while Fraction C showed a ratio of 1:1 indicative of a glycerol teichoic acid. This latter fraction also was associated with glucose, galactose, alanine, and fatty acids. Diglycerol triphosphate, the compound characteristically released from 1-3 phosphodiester-linked glycerol teichoic acids by alkaline hydrolysis, was isolated and characterized. Alanine was identified as its alkaline-labile, ester-linked D-isomer. A glyceride was isolated containing a disaccharide of equimolar glucose and galactose attached to the 2-hydroxyl group of glycerol. Hapten inhibition analysis demonstrated that β-galactosides were the greatest

inhibitors (>75%) while glucose and its derivatives inhibited to a much lesser extent (<30%). Comparative immunodiffusion and immunoelectrophoresis analyses demonstrated that all six Bratthall group *a* organisms tested contained this antigenic determinant and that it was absent in groups *b*, *c*, and *d*. It is suggested that the common antigenic determinant of this serologic group within *S. mutans* may be a β-galactoside associated with a glycerol teichoic acid and possibly other polymers.

INTRODUCTION

The classification and identification of the various strains of *Streptococcus mutans* have eluded investigators until recently. One of the more promising methods introduced for grouping these organisms has been the application of serology. Preliminary work by investigators in this and other laboratories has indicated that a serological classification scheme is plausible. In this dissertation I will be concerned with defining the immunochemical basis of the first serological group. A possible group-specific determinant was isolated from the membrane of *S. mutans* AHT, a group *a* organism, and the biochemical and immunochemical characterization of this polymer as a membrane-bound glycerol teichoic acid will be presented herein.

Pathogenesis of S. mutans--Since the earliest days of bacteriological studies, investigators have believed that the microorganisms found in the mouth were related in some way to dental decay. Leber and Rottenstein (72) described microorganisms in carious dentine as early as 1873. Streptococci were reported in the oral cavity by Miller (68) who demonstrated in 1890 that bacteria were the etiological agents of dental decay. By incubating a

sterile extracted tooth in a mixture of human saliva and bread, he observed that bacteria fermented the carbohydrates, thereby producing acids to decalcify the tooth. Goadby (45) expressed the opinion that the combined action of two types of microorganisms, the "acid-producers" and the "dentine-liquefiers," was the cause of carious lesions.

Clarke (20) experimented with a small, pleomorphic, acidogenic organism isolated from 36 of 50 carious teeth. The organism caused the initiation of caries in a sterile tooth incubated in glucose broth. In plain broth media, the organism appeared as a chained coccus, while on glucose agar and in acid broth it appeared as a coccobacillus and therefore he named this organism *S. mutans*. Maclean (62) reportedly confirmed Clarke's observations by isolating a similar organism from the dentine of carious teeth.

Not until 1955 and the work of Orland *et al.* (72) did more sophisticated experimentation allow advances in this field. Up until that time, studies of the pathogenesis of tooth decay were confused by the presence of various kinds of microorganisms around the teeth even in the absence of decay. Orland and his co-workers employed germfree (or gnotobiotic) experimental rats, and they attempted to determine which organism(s) might be capable of producing typical carious lesions. Streptococci and other organisms isolated from carious rat molars were used as inocula. One set of gnotobiotic rats was inoculated with a combination

of an enterococcus and an anaerobic pleomorphic rod while another set was inoculated with the same enterococcus and a proteolytic aerobic rod. The organisms were applied by swabbing the rats' teeth surfaces with bacterial suspensions. All the rats developed extensive caries after introduction of the populations of bacteria. In all experiments, rats were fed a high carbohydrate diet. A control sample of uninoculated rats and monoinfected with either the proteolytic bacillus or the anaerobic rod did not develop caries even though they were fed the caries-producing diet. This set of experiments indicated that dental caries is primarily a bacterial disease and involves a streptococcal species. In 1959, Orland (71) repeated his experiments with pure cultures of the enterococcus and again demonstrated a member of the genus *Streptococcus* as the etiological agent of dental decay. The enterococcus was isolated from a carious rat molar and resembled *S. faecalis*.

Fitzgerald and Keyes (42), Keyes (54), and Fitzgerald *et al.* (41) repeated Orland's work using streptomycin-resistant strains as their inocula for use in rats and hamsters. Streptococci were isolated from carious lesions and a streptomycin-resistant mutant of each organism was selected by standard genetic techniques. These organisms either singly or pooled were inoculated into caries-free hamsters and rats by the technique of Orland and later could be isolated from the animal's oral cavity, feces,

and carious lesions upon feeding the animals a high-sugar diet. These workers also demonstrated that dental decay is a transmissible disease by placing caries-free hamsters in cages with experimentally induced caries-active hamsters. After 35 days, most hamsters were caries-active and the streptomycin-resistant strain employed was isolated from all carious lesions. Streptomycin-resistant strains could not be isolated from uninfected caries-free hamsters. Transmission was by ingestion of fecal material since caries-free hamsters became caries-active after being fed feces obtained from caries-active hamsters. Implantation of the cariogenic flora was retarded by the action of the antibiotics penicillin and erythromycin, thereby supporting the notion that a gram-positive organism was involved.

Orland (72), Fitzgerald and Keyes (42), and Fitzgerald *et al.* (41) expressed the opinion that caries-producing organisms are host-specific with regard to cariogenicity. But Zinner *et al.* (92) isolated cariogenic streptococci from human carious lesions and showed that they are related to rat and hamster strains by the use of fluorescein-labeled antisera. Furthermore, human strains inoculated into the oral cavity of rats and hamsters caused rampant caries. Krasse (58) also isolated streptococci from human caries. He judged these to be similar or identical to the cariogenic streptococci by their biochemical and morphological characteristics and was able to induce caries in

hamsters. Gibbons *et al.* (43) performed similar implantations in rats with human cariogens using these organisms. Krasse *et al.* (59), using human subjects, was able to implant either human or hamster strains into their oral cavities. It can be concluded from the above that dental caries is a transmissible bacterial disease caused by acidogenic streptococci which do not demonstrate specificity of infection.

One of the physiological characteristics of cariogenic streptococci is their production of large quantities of non-dialyzable extracellular capsule from sucrose (43). Wood and Critchley (90) acid-hydrolyzed isolated samples of capsular material and demonstrated that the hydrolysis products were glucose and fructose derived from dextran and levan polymers, respectively. Plaque formation was shown to depend upon the presence in the oral cavity of streptococci which are able to synthesize these extracellular polysaccharides (40). These polysaccharides, which are synthesized by the enzymes dextransucrase and levansucrase (34), confer upon the bacterium the capability of adhering to tooth surfaces. Both of these enzymes are apparently present on the surface of the bacterial cell and are produced constitutively (19, 26).

Krasse (57) implanted streptomycin-resistant, caries-inducing streptococci into the oral cavity of hamsters which had been fed various diets. He determined that the composition of the diet was a main factor in the implantation of

the organisms. The standard powdered caries test diet (sucrose and skim milk) aided in the implantation of the caries-producing organisms while the standard commercial animal diet gave a low number of implanted organisms. Also, comparing sucrose- versus glucose-supplemented diets (56), he demonstrated high implantation and high caries production in sucrose-fed hamsters while glucose-feeding gave low implantation and low caries production. In their work with human subjects, Krasse *et al.* (59) found that a higher degree of implantation of cariogenic streptococci occurred if the subjects chewed a substance with sucrose included. These experiments further indicated that the polysaccharide capsules produced by these organisms from sucrose aid in their attachment to tooth surfaces and in eventual caries production.

Further experiments by Gibbons and Fitzgerald (44) demonstrated that washed cells of *S. mutans* agglutinated on addition of high molecular-weight dextrans to the medium. This phenomenon also occurred when sucrose was added to the medium, indicating that it was rapidly converted by cell-bound dextransucrase into dextran. Fluorescein-labeled cells of *S. mutans* adhered to dextran-containing plaque and also dextran-treated teeth. A mutant which had lost its ability to adhere to teeth (29) when implanted into germfree rats was unable to initiate dental caries. This mutant otherwise was identical to *S. mutans* strains. Therefore,

it would appear that the ability to form an insoluble polysaccharide which allows adhesion to the surfaces of teeth and possibly to other organisms is an important factor in the etiology of dental caries.

Evidence has been presented that dental caries is a bacterial disease which is readily transmissible in test animals. Acidogenic streptococci which form adhesive dextran capsules appear to be the etiological agents in dental decay. The ability to rapidly produce large amounts of acid from glucose in a localized area on the tooth surface is definitely the initiating process in carious lesions. A chronic decalcification of enamel results from acid production. Since they are proteolytic, it is believed these organisms also destroy the layer immediately under the enamel, the dentine, thereby opening a pathway into the pulp and eventually the blood stream (70).

Classification of S. mutans--"Facts in isolation amount to mere gossip; facts in relation become philosophy." This quote by Justice Oliver Wendell Holmes very nicely sums up the state of the classification of the cariogenic streptococci. Unfortunately, the "facts in isolation" have dominated over the past eighty years and we are just now beginning to enter the phase of "facts in relation."

Gordon (47) introduced the first method for differentiating streptococci in 1905. He isolated 300 streptococci

from human saliva and identified them as 48 different types by a series of nine biochemical tests. The tests included the clotting of litmus milk, the anaerobic reduction of neutral red broth, the production of acid end-products, and the fermentation of seven different sugars. In 1906, Andrewes and Hordes (2) enlarged Gordon's regimen of biochemical assays and tested 1,200 isolated colonies. They described the oral streptococci *S. mitis* and *S. salivarius* as well as other recognized species of streptococci. Clarke (20) is recognized as the first person to isolate and completely describe a cariogenic streptococcus which he named *S. mutans*.

In 1933, Lancefield (60) placed 106 strains of *S. haemolyticus* into five different serological groups using the precipitin reaction. Only two of the 106 strains were unclassifiable. Using hot acid extracts of whole cells, Lancefield originated the first five groups (A-E) of her now-famous grouping scheme for streptococci.

Sherman (78) believed that the task of grouping streptococci could be made less chaotic by first placing the organisms into divisions and then separating them by their biochemical, morphological, and serological properties. Using the organisms' growth temperature optima and limits, tolerance to salt and alkali, and hemolytic properties as criteria for division, Sherman divided the streptococci into four main divisions. The divisions were named the

enterococcal, lactic acid, pyogenic, and viridans groups. The cariogenic and other oral streptococci are placed mainly in the viridans group which unfortunately has become the haven for many unclassifiable streptococci.

In 1960, Fitzgerald and Keyes (42) and Fitzgerald *et al.* (41) isolated various cariogenic streptococci from the oral cavities of rats and hamsters. They were designated HS-(hamster) strains and FA-(rat) strains. They noted that the fermentation of sorbitol distinguished the cariogenic streptococci and that extracts of these organisms would not give a precipitin reaction with antisera designating the various groups (A-H, K-S) of the Lancefield serological scheme. Also, these organisms cannot be classified according to the scheme for streptococci in Bergey's Manual of Determinative Bacteriology. They believed these isolates belonged "somewhere between the enteric and lactic groups." Bergey's Manual recognizes 19 species of classified streptococci and maintains 7 species, unnamed and unclassified, whose relationship to the classified streptococci is unknown. Zinner *et al.* (92) isolated various cariogenic streptococci (AHT, BHT, CHT, and HHT) of human origin. These organisms also could not be classified by the Lancefield scheme. Carlsson (17) noted the presence of the above non-hemolytic streptococci in dental plaque of man and reintroduced use of the nomenclature previously used by Clarke (i.e., *S. mutans*). Since Clarke's original strains were lost, it is impossible

to determine if the *S. mutans* of Carlsson and others are those of the original worker.

Slade and Slamp (80) isolated carbohydrate polymers from the cell walls of organisms representing 17 different Lancefield groups. The sugars most commonly found were rhamnose, glucose, galactose, arabinose, and mannose. They believed the unclassifiable streptococci could eventually be grouped by the qualitative and quantitative analysis of their cell-wall carbohydrates since the main somatic antigens of most streptococci are cell-wall localized. Groups D and N, however, have membrane-bound teichoic acids as group antigens.

Colman and Williams (21) subjected 216 strains of nonhemolytic streptococci to cluster analysis. The dextran-producing streptococci formed a separate single cluster which was composed basically of *S. mutans*. Using 20 biochemical tests, Guggenheim (48) grouped 86 plaque isolates into 44 biotypes. He also defined *S. mutans* as a homogeneous group of organisms. Drucker *et al.* (32) analyzed the amino acid composition of cell walls obtained from both cariogenic and non-cariogenic streptococci and found no differences of any significance. Streptococci isolated from dental plaque and identified by their colonial morphology by Edwardsson (35) also formed a homogeneous group when their physiological properties were tested. Drucker and Melville (30, 31) and Carlsson (18) demonstrated that *S. mutans* strains formed

a distinct group by means of numerical taxonomic methods. The other oral streptococci, *S. sanguis*, *S. mitis*, and *S. salivarius*, did not fall into this grouping.

Recent publications indicate that the organisms classified as *S. mutans* are actually quite heterogeneous, both genetically and serologically. Jablon and Zinner (53, 91) placed 8 strains of oral streptococci into four serological groups by means of immunofluorescence. Groups I and II contained various human, rat, and hamster cariogenic streptococci while groups III and IV contained only one oral streptococcal strain each. They were unable to eliminate serological cross-reactions against other streptococci, thereby presenting a problem in using this scheme for classifying oral streptococci. This technique was useful, though, in identifying some of the more pathogenic human cariogens.

Using the 116 strains from Carlsson's (18) numerical taxonomic studies, Bratthall (12) divided *S. mutans* into three distinct serological groups (*a*, *b*, and *c*). He also was hampered by numerous cross-reactions but could show the three distinct group-specific antigenic reactions using comparative immunoelectrophoresis. In 1970, Bratthall (13) repeated his experiments with 70 strains of *S. mutans* and was able to place 58 of them into his groups *a*, *b*, and *c*. By comparative immunoelectrophoresis, Bratthall demonstrated two new groups (*d* and *e*) into which the remaining 12 strains

were placed. His groups *a* and *b* correspond to Jablon and Zinner's groups I and II while the organisms of his group *e* reacted against antisera to Lancefield group E. The four other groups showed no cross-reactions with any of the 17 Lancefield groups.

Recently, Bratthall (14) produced fluorescein-labeled antisera and after specific adsorptions he eliminated all cross-reactions against groups *a*, *b*, and *d*. Group *e* antisera reacted with both group *e* and Lancefield group E organisms. By adsorbing Lancefield group E antisera with a group *e* organism, he prepared antisera which would only react with Lancefield group E organisms. Therefore, an organism reacting with group *e* antisera and not with adsorbed Lancefield group E antisera belonged in Bratthall's group *e*, while an organism reacting with both antisera belonged in Lancefield group E. Bratthall was unable to produce antisera which reacted only against group *c* and not group *e* as well. Therefore, if an organism reacts with both groups *c* and *e* antisera it belongs to group *c*. Another way to check this would be to isolate the organism in question, prepare a Lancefield extract, and run comparative immunoelectrophoresis which would demonstrate to which group the organism belongs, since Bratthall demonstrated that each of the group-specific antigens migrated in a different manner. Using the above-mentioned fluorescein-labeled antisera, Bratthall (14) demonstrated members of all five

serological groups exist in the mouths of individuals sampled in regions all over the world.

Coykendall, through deoxyribonucleic acid (DNA) base composition studies (23) and DNA-DNA reassociation (hybridization) experiments (24, 25), placed strains of *S. mutans* into four genetic groups. The guanosine-cytosine (GC) content of his four groups (I, II, III, and IV) was 37-38%, 42-43%, 44-45%, and 43-44%, respectively. Remarkably, Coykendall's four genetic groups (I-IV) related to Bratthall's serological groups *c*, *b*, *d*, and *a*, respectively. De Stoppelaar (28) divided the cariogenic streptococci into only three serological groups (MI, MII, and MO) by precipitin analysis. These groups correspond to Bratthall's groups *c*, *a*, and *e*.

The above work of Jablon and Zinner, Bratthall, de Stoppelaar, and Coykendall subdivided *S. mutans* into five groups of cariogenic organisms by genetic and serologic criteria. Bleiweis *et al.* (9) further demonstrated that the organisms of groups *a* and *b* have dissimilar cell-wall compositions. Both groups contain rhamnose, glucose, and galactose to varying degrees, but the group *a* organism AHT contains threonine while the group *b* organism BHT does not contain threonine in its wall, but instead contains more alanine and probably a glycerol teichoic acid.

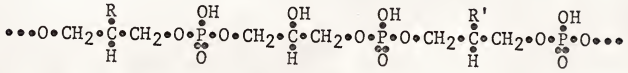
Classification by means of teichoic acid antigens--Repeating units of glycerol phosphate linked by 1-3 phosphodiester bonds comprise the backbone of a glycerol teichoic acid as shown in Figure 1A (3, 4, 6). This skeletal structure by itself was shown to be weakly antigenic by McCarty (65). Upon alkaline hydrolysis, a 1-3 phosphodiester-linked glycerol teichoic acid is degraded to glycerol, glycerol phosphate, glycerol diphosphate, and a unique compound characteristic of the 1-3 phosphodiester linkage, diglycerol triphosphate (3, Fig. 1B). Since teichoic acid can be degraded from both ends and the terminal phosphate groups can cyclize with the 2-hydroxyl position of glycerol, the diglycerol triphosphate will be formed because adjacent hydroxyl groups must be present for further hydrolysis (15).

The free 2-hydroxyl group of the glycerol moieties can be substituted with D-alanine and/or various glycosyl substituents (see Fig. 1A). All polymers isolated to date contained some D-alanine, which was shown by McCarty (66) to be weakly antigenic.

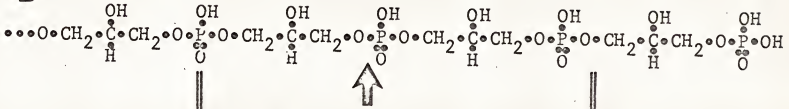
The immunological specificity of the polymer is usually inherent in the qualitative and quantitative natures of its glycosidic side-groups as well as their anomeric linkages (see Table 1). Teichoic acids have been demonstrated to be group-specific antigens in the genera *Streptococcus, Lactobacillus,* and *Staphylococcus*. The streptococcal group D antigen (87) is a membrane-bound

Fig. 1. Structure of a 1-3 phosphodiester-linked glycerol teichoic acid and its hydrolysis by alkali. A, Polymer with D-alanyl (R) and glycosyl (R') side groups; B, Alkaline hydrolysis and its products. The arrow indicates a possible point of hydrolysis for the formation of diglycerol triphosphate.

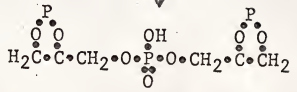
A



B



GLYCEROL
GLYCEROL PHOSPHATE
GLYCEROL DIPHOSPHATE



DIGLYCEROL TRIPHOSPHATE

Table 1. The antigenic teichoic acids

GENUS	GROUP	TYPE AND LOCATION	SIDE GROUP	REFERENCE
Streptococcus	D	glycerol (m^a)	kojibiosyl	Wicken and Baddiley (86)
	N	glycerol (m)	galactosyl	Elliott (36)
Lactobacillus	A	glycerol (m, w^b)	glucosyl	Knox and Wicken (55)
	D	ribitol (w)	glucosyl	Sharpe *et al.* (75)
	E	glycerol (w)	glucosyl	Sharpe *et al.* (75)
	F	glycerol (m)	glucosyl, galactosyl	Wicken and Knox (88)
Staphylococcus	A	ribitol (w)	N-acetylglucosaminyl	Baddiley *et al.* (7)
	B	glycerol (w)	N-acetylgalactosaminyl	Ellwood *et al.* (37)

[a]membrane

[b]wall

glucosyl-glucosyl glycerol teichoic acid (83, 86) and the group N antigen is a membrane-bound galactosyl glycerol teichoic acid (36). The lactobacilli group A antigen is a combination of both wall- and membrane-bound glucosyl glycerol teichoic acids while the group F antigen is a membrane-bound galactosyl-glucosyl glycerol teichoic acid (75, 89). Both groups D and E antigens are wall-associated teichoic acids, the D antigen being a glucosyl ribitol teichoic acid and the E antigen a glucosyl glycerol teichoic acid (75, 89). Wall-associated teichoic acids also act as group antigens for groups A and B staphylococci. The group A polysaccharide is an N-acetylglucosaminyl ribitol teichoic acid and the group B polysaccharide an N-acetylgalactosaminyl glycerol teichoic acid (27, 51).

The existence of glycerol teichoic acids on membranes of all gram-positive bacteria has been postulated by Baddiley (6). Hay *et al.* (52), Shattock and Smith (76), and Slade and Shockman (79) formed protoplasts of gram-positive organisms and demonstrated that about 85% of the glycerol teichoic acid was found outside the cell membrane in the periplasmic space. The remainder of the teichoic acid was found with the membrane fraction upon lysis. Very small amounts of nucleic acids were released during the protoplasting process; therefore, it would appear the teichoic acid binds to the outer surface of the cell membrane since only minor amounts of internal constituents

were released. Wicken and Knox (89) and Toon *et al.* (83) isolated "lipoteichoic acids" from lactobacilli and streptococci, respectively, confirming the linkage of the teichoic acid to the cell membrane. The glycerol phosphate chain is covalently linked to a diglycosyl diglyceride, the main diglyceride of the bacterial cell membrane (83).

MATERIALS AND METHODS

Bacterial strains and growth conditions--The cariogenic organisms used in this study are listed in Table 2 according to their serological group.

Stock cultures were maintained in agar stabs and as lyophilized cultures. The organisms were grown for 12 hr at 37 C in Todd-Hewitt broth (Difco Laboratories, Detroit, Michigan), pH 7.0, containing 0.5% glucose (THG) and inoculated into agar stabs of THG. The agar stabs of THG contained 2.0% Bacto-Agar (Difco) and 1.0% calcium carbonate. Lyophilized stocks were prepared by resuspending the 12-hr cultures in sterile 20% skim milk (Pet Inc., St. Louis, Missouri) and sealing the ampoules under vacuum after lyophilization with a Virtis freeze-dry unit (Virtis Research Equipment Co., Gardiner, New York). Ten-liter batches of strain AHT were grown for 20 hr at 37 C in THG and harvested in a Delaval gyro-tester (Delaval Separator Co., Poughkeepsie, New York). These organisms served as the source of crude extract.

Extract preparation--Extracts were prepared basically as described by Wicken and Knox (88). Cells harvested in a gyro-tester were washed three times in saline and broken in

Table 2. Organisms used and their origins

ORGANISM	ORIGIN	REFERENCE
*S. mutans a**		
AHT	Human	Jablon and Zinner (53)
3720, E49, HS6, HS1	Hamster	Fitzgerald and Keyes (42)
OMZ 61	Rat	Guggenheim *et al.* (49)
S. mutans b		
BHT	Human	Jablon and Zinner (53)
FA1	Rat	Fitzgerald *et al.* (41)
S. mutans c		
JC2	Human	Carlsson (17)
NCTC 10449	Human	Edwardsson (35)
GS5	Human	Gibbons *et al.* (43)
S. mutans d		
B13	Human	Edwardsson (35)
OMZ 176	Human	Guggenheim (48)
SL1R, K1R	Human	Fitzgerald (39)

**S. mutans* strains are grouped according to Bratthall (13).

a Braun Tissue Homogenizer (11). Whole cells and walls were collected by centrifugation at 12,000 x g for 20 min in a Sorvall RC2-B (Ivan Sorvall Inc., Norwalk, Connecticut) and the supernatant was centrifuged further at 78,000 x g for 2 hr in a Beckman Model L ultracentifuge (Beckman Instruments, Palo Alto, California). The later supernatants were pooled and lyophilized. The crude material (6% w/v) was stirred with cold 10% trichloroacetic acid (TCA) for 24 hr at 4 C and the precipitated material was collected by centrifugation at 20,000 x g for 30 min and re-extracted. The pooled extracts were shaken with ether (3 vol) to remove TCA from the aqueous fraction until a final pH of 4.0 was attained in the aqueous fraction; the ether was discarded. Finally the sample was dialyzed for 12 hr against 200 vol of distilled water at 4 C after which it was lyophilized. This material served as the crude extract for agarose chromatography.

Agarose chromatography--Columns (2.7 x 90 cm) of Biogel A-5m (Biorad Laboratories, Richmond, California) were poured and equilibrated with 0.2 M ammonium acetate buffer (pH 6.9) at 4 C. A Beckman Model RC 16B2 conductivity bridge (Beckman Instruments) was used to monitor the ionic strength of the equilibrating buffer. After the columns were equilibrated, samples were loaded onto the column and eluted using the above-mentioned ammonium acetate buffer as

described by Wicken and Knox (88). Flow rates of 8-9 ml/hr were maintained and fractions (2.5 ml) were collected in a Gilson Mini-Escargot fraction collector (Gilson Medical Electronics Inc., Middleton, Wisconsin). Fractions were analyzed for phosphorus as described below and for 260 nm absorbing material using a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Alkaline hydrolysis and diethylaminoethyl (DEAE)-cellulose chromatography--Samples (25 mg) were hydrolyzed in sealed ampoules for 3 hr at 100 C in 1 M sodium hydroxide (NaOH) (4 ml). The hydrolysates were passed through a column (1 x 12 cm) of Ag 50W-X8 (hydrogen form) resin (Biorad) and the eluates were adjusted to pH 7.0 with 0.5 M ammonium hydroxide (5).

A column (1.5 x 27 cm) of Whatman DE 32 (1.0 meq/ml) (Reeve Angel, Clifton, New Jersey) was prepared according to Whatman Information Leaflet IL2 and equilibrated with water. The neutralized alkaline hydrolysates were loaded onto the column, followed by partial elution with 200 ml of water as described by Archibald *et al.* (5). The samples were further eluted using a linear gradient of 0.0-0.2 M ammonium carbonate buffer (pH 8.9). Fractions (2.5 ml) were collected in a Gilson Mini-Escargot fraction collector. Fractions were assayed for phosphorus (see below) and the phosphorus-containing tubes were pooled according to peak areas and lyophilized.

Quantitative assays--Phosphorus was determined by the method of Lowry *et al.* (61). In this procedure an ashing mixture composed of perchloric and sulfuric acids is added to liquid samples. The samples are then dried at 95 C for 2 hr after which they are ashed at 165 C for an additional 2 hr. Finally a mixture of ascorbic acid in ammonium molybdate-sodium acetate buffer is added to the samples which are then incubated at 37 C for 2 hr. The blue color of the reduced phosphomolybdic acid is then read at 820 nm.

Glucose and galactose were determined using the Glucostat and Galactostat reagents (Worthington Biochemical Corp., Freehold, New Jersey). Each of the reagents catalyzes the oxidation of the sugar in the presence of the specific oxidase with the formation of hydrogen peroxide. The newly formed peroxide then oxidizes a reduced chromagen in the presence of a peroxidase. The color of the oxidized chromagen is then read at its specific wavelength. Samples for glucose and galactose determinations (8-10 mg) were hydrolyzed in sealed ampoules for 4 hr at 100 C in 2 M hydrochloric acid (HCl) (3 ml). Hydrolyzed samples were washed three times with 1.0 ml aliquots of water and neutralized with 5 M NaOH and 0.1 M HCl using phenolphthalein as indicator. Neutralization occurred at a volume of 7.6-7.8 ml and the sample volume was brought up to 10.0 ml with 0.266 M sodium phosphate buffer (pH 7.0). The samples were assayed according to the procedures outlined in the Glucostat (1971) and Galactostat (1966) information leaflets except

for one alteration. In both assays the Glucostat and Galactostat vials were brought to volume using 0.067 M sodium phosphate buffer (pH 7.0).

Glycerol was determined using the Glycerol Stat-Pack (Calbiochem, Atlanta, Georgia) according to the instructions in information leaflet 3056. The Glycerol Stat-Pack measures glycerol using the coupled enzymatic reactions of glycerol kinase, pyruvate kinase, and lactate dehydrogenase. The amount of glycerol in the sample is measured by the loss of color at 340 nm due to the oxidation of nicotinamide adenine dinucleotide. Samples for glycerol determinations (10 mg) were hydrolyzed in sealed ampoules for 3 hr at 100 C in 2 M HCl. The acid was removed by means of lyophilization of hydrolysates, followed by three washings of each sample with subsequent lyophilization. Glycerol phosphate and glycerol diphosphate, products of acid hydrolysis, were then dephosphorylated with alkaline phosphatase (Worthington) to yield free glycerol. Washed hydrolyzed samples were incubated in sealed ampoules for 24 hr at 37 C in 0.05 M ammonium carbonate buffer (pH 9.5) in the presence of alkaline phosphatase (2 mg). These preparations served as sources for the glycerol assay.

Amino acids and amino sugars were determined using a JOEL Model JLC-5AH automated amino acid analyzer (JEOL, Inc., Cranford, New Jersey) according to Spackman *et al.* (81). Samples for amino acid and amino sugar analysis

(7 mg) were hydrolyzed in sealed ampoules for 11 hr at 105 C in 4 M HCl (5 ml). The acid was removed using a rotary flash evaporater (Buchler Instruments, Fort Lee, New Jersey) and the residues were washed three times with water (5 ml). The washed residues were brought to volume (20 ml) in 0.1 M HCl.

Paper and thin-layer chromatography--The following solvent systems were used: A, n-propanol + aq. ammonia (sp.gr. 0.88) + water [6:3:1 by vol., Whatman #4 ascending (50)], and B, ethyl acetate + pyridine + water [10:4:3 by vol., Whatman #1 descending (46)]. Chromatograms to be eluted were run as bands on Whatman #3MM paper previously washed with 2 M acetic acid followed by water.

Products were detected on papers by the following spray reagents wherever appropriate: periodate, benzidine, and silver nitrate for polyols and glycosides (85); alkaline silver nitrate for sugars (84) and modified for glycosides (16); molybdate for phosphoric esters (50); ninhydrin for amino acids (22) and Morgan-Elson reagent for amino sugars (38).

For thin-layer chromatography, Gelman ITLC type SA chromatography media (Gelman Instrument Co., Ann Arbor, Michigan) were used. Sheets were prepared for chromatography as described by Bleiweis and Coleman (8). Chromatograms were developed in light petroleum (b.p. 40-60

C) + ethyl ether + acetic acid [80:20:1 by vol. (88)] and sprayed with dichlorofluorescein, aluminum chloride, and ferric chloride for fatty acids (33).

Analytical ultracentrifugation--Sedimentation velocities of purified fractions were determined using a Model E ultracentrifuge (Beckman). Samples (10 mg) in saline were centrifuged at 59,780 rpm for 128 min at 20 C. S_{20} values were calculated according to the method of Schachman (74).

Ammonolysis and configuration of released products--Samples (2 mg) were shaken with 1.0 ml of methanol-aq. 2 M ammonia (1:1 v/v) for 3 hr at room temperature. Samples were chromatographed in solvent B and products were detected with the ninhydrin spray reagent (see above).

Similar chromatograms were sprayed with D-amino acid oxidase (Sigma Chemical Co., St. Louis, Missouri) and incubated for 6 hr at 37 C in a sealed moist oxygenated chamber (86). After the chromatograms dried, they were sprayed with 2,4 dinitrophenylhydrazine in HCl (69) to demonstrate the presence of keto acids derived from D-amino acids. The same procedure was carried out using L-amino acid oxidase (Sigma).

Preparation of rabbit antisera--Organisms to be used for vaccinations were grown (20 hr) in centrifuge bottles containing 250 ml THG. The cells were pelleted by

centrifugation and washed two times with sterile saline. After resuspension in 100 ml of 0.6% formalin, the organisms were incubated for 24 hr at room temperature. They were washed two times with saline and diluted to a reading of 360 using a #66 filter on the Klett-Summerson colorimeter (Klett Instrument Co., New York, New York). The cells were pelleted again and resuspended in one-tenth their original volume. This suspension served as the source of the inoculum.

On Monday, Wednesday, and Friday of the first week, New Zealand white male rabbits were injected intravenously with 0.1 ml of vaccine. This regimen was repeated with 0.2 ml the second week and 0.3 ml the third week. On Monday of the fourth week, the rabbits were bled from the ear artery and the sera were titrated by capillary precipitin reactions (82). Rabbits whose sera exhibited a 4+ reaction were bled from the heart (40 ml) on two alternate days and sacrificed on the third after a final bleeding. The antisera were stored at 4 C after the addition of sodium azide (0.01%).

Preparation of mild Lancefield extracts--Extracts to be used for serological studies were prepared by modifying the Lancefield technique (60). Cells were grown in centrifuge bottles containing 250 ml THG for 18 hr at 37 C. The cells were pelleted and washed one time with saline. Cells were finally resuspended in saline (2 ml) and 4 M HCl was added until a final pH of 2.5 was reached. The

suspensions were then placed in a boiling water bath for 10 min and then cooled quickly. Finally the cells were pelleted, discarded, and the supernatants were neutralized with 0.077 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaOH. These preparations then served as the mild Lancefield extracts for serological studies.

Quantitative precipitin assay--Antigen in saline containing 0.02 M ethylenediaminetetraacetic acid (s-EDTA) (pH 7.0) was added to an equal volume of antisera (0.3 ml) and incubated for 1 hr at 37 C after which the tubes were placed for 4 days at 4 C. The precipitates were collected at room temperature by centrifugation at 1550 rpm for 10 min in a Precision Clinical Centricone (Precision Scientific Co., Chicago, Illinois) and washed three times with 1.0 ml s-EDTA. The washed precipitates were dissolved and brought up to final volume (1.0 ml) with 0.1 M NaOH. Absorption at 280 nm was read on a Gilford Model 240 spectrophotometer and the mg protein/ml values were determined from a standard curve using rabbit gamma-globulin (Calbiochem) as a standard. All samples were run in triplicate.

Quantitative precipitin inhibition assay--Each compound to be tested (75 µmoles) was incubated with 0.3 ml antisera (dil. 1:10). A standard was run with 0.3 ml antisera and 0.1 ml s-EDTA. Each of the assay tubes was brought up to 0.4 ml with s-EDTA (all tubes were run in

duplicate). After 30 min incubation at 37 C, 25 µg of antigen were added to each tube and incubation continued for an additional hr. Finally the tubes were placed for 5 days at 4 C and precipitates assayed for protein contents (see quantitative precipitin assay).

Gel diffusion and immunoelectrophoresis--Slides for gel diffusion precipitin tests (63) were prepared by dipping clean microscope slides into molten 0.2% Ionagar #2 (Colab, Chicago Heights, Illinois). The slides were allowed to dry with a thin film of agar. A mixture (2.5 ml) containing 0.8% ionagar, 0.8% sodium chloride, 0.02 M sodium phosphate buffer (pH 7.0), and 0.001% sodium azide was pipetted to uniformly cover the slide surfaces. The slides were allowed to harden in a moist chamber until they were ready to be used for gel diffusion analyses by the Ouchterlony technique. Wells were cut using a Pasteur pipette with a rubber bulb for removal of excess gel. The wells were filled with the appropriate antigen solution or antiserum using coagulation capillary tubes.

Slides for immunoelectrophoresis (13) were prepared by basically the same techniques as for the gel diffusion slides except that a 0.05 M sodium barbital-HCl buffer (pH 8.2) was used. Wells and troughs were punched out using a Shandon gel cutter (Colab). Antigen solution was added to the wells and the slides were placed into a Shandon immunoelectrophoresis apparatus (Colab) with a LKB Model

3371 power supply (LKB-Produkter AB, Stockholm-Bromma, Sweden) and run for 70 min at a constant current of 10 ma per slide. Finally the troughs were filled with antisera and the slides were incubated at 4 C until the precipitin bands appeared.

Chemicals--Organic solvents and acids and most common salts were obtained from Mallinckrodt (Scientific Products, Chamblee, Georgia). Amino acids and amino sugars as well as benzidine, 2,4-dichlorofluorescein, glycerol phosphate, glycerol diphosphate, α-methyl glucopyranoside, ß-methyl glucopyranoside, and α-methyl galactopyranoside were obtained from Sigma Chemical Co. D-glucose and D-galactose were obtained from Calbiochem. ß-methyl galactopyranoside was obtained from Schwarz/Mann, Orangeburg, New York, while ninhydrin was obtained from Pierce Chemical Co., Rockland, Illinois.

RESULTS

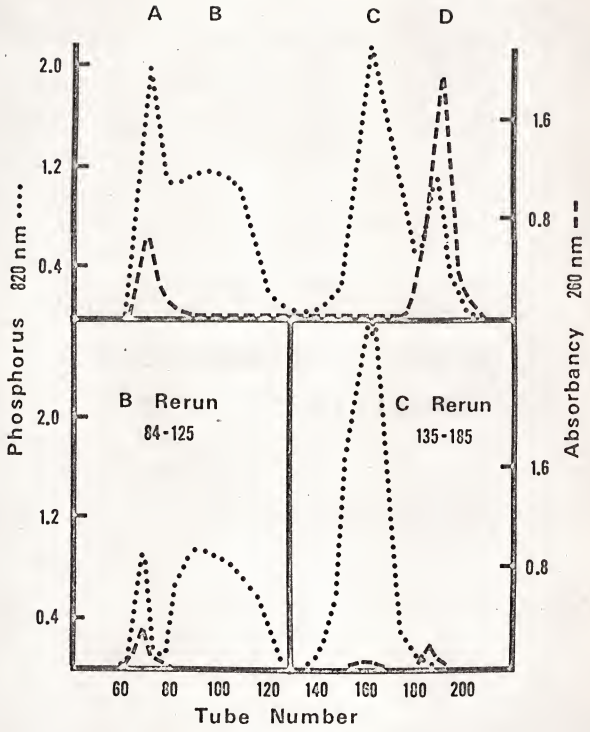
Preliminary observations--Preliminary evidence from studies in our laboratory indicated that *S. mutans* BHT, a Bratthall group *b* organism, contained a glycerol teichoic acid as part of its cell wall. A similar polymer was absent from the walls of *S. mutans* AHT (9). Since streptococcal group-specific antigens have been demonstrated to occur associated with the membrane in the Lancefield groups D and N streptococci, it was possible a similarly situated antigen was present in *S. mutans* AHT. We suspended whole cells of *S. mutans* AHT in 0.066 M potassium phosphate buffer (pH 7.3) for 10 min in a boiling water bath according to the method of Prytz and Jablon (73) in order to release membrane-associated antigens. Whole cells were removed by centrifugation for 15 min at 1550 rpm in a Precision Clinical Centricone. The neutralized supernatant reacted strongly with Bratthall group *a* antisera indicating that the group-specific antigen might be associated with the membrane.

The supernatant was lyophilized and acid hydrolyzed according to the method used for preparation of samples for glycerol determinations (Material and Methods). The volume of the final washed sample was brought up to 1.0 ml

with water. The hydrolysate was analyzed by paper chromatography using solvent systems A and B followed by each of the spray reagents. Glycerol, glycerol phosphate, as well as amino acids and sugars were found to be present, indicating that possibly a membrane-associated glycerol teichoic acid was present and could be the group-specific antigen. The method of Wicken and Knox for the isolation of membrane-associated glycerol teichoic acids (88) was adopted as our basic procedure and is outlined in Materials and Methods.

Preliminary qualitative analysis and gel chromatography--Crude extracts (10 mg) obtained by TCA extraction were hydrolyzed in sealed ampoules for 3 hr at 100 C in 2 M HCl (2 ml). The acid was removed by lyophilization, the samples were neutralized, and the hydrolysates were chromatographed in solvent systems A and B. Compounds were detected by various spray reagents (Materials and Methods). Glycerol, glycerol phosphate, glycerol diphosphate, alanine, glucose, and galactose were the major products. Fatty acids were also shown to be present. Standards were co-chromatographed with the unknowns to identify the various compounds. These compounds again were indicative of a membrane-associated glycerol teichoic acid. Crude extract (150 mg) was loaded onto a 6% agarose column and four phosphorus-containing peaks (A, B, C, and D) were resolved (Fig. 2). Fractions 84-125 and 135-185 were pooled separately, lyophilized, and rerun in order to further exclude

Fig. 2. Separation of crude extract derived from *S. mutans* AHT on a 6% agarose column. Samples (150 mg) were eluted using a 0.2 M ammonium acetate buffer (pH 6.9) at 4 C. Flow rates of 8-9 ml/hr were maintained and fractions (2.5 ml) were collected. Fractions 84-125 and 135-185 were collected, pooled separately, lyophilized, and rerun for further separation. A, B, C, and D signify the four phosphorus-containing fractions.


A
B
C
D
2.0
1.2
0.4
1.6
0.8
Phosphorus 820 nm
Absorbancy 260 nm
B Rerun
84-125
C Rerun
135-185
2.0
1.2
0.4
1.6
0.8
60
80
100
120
140
160
180
200
Tube Number

any 260 nm absorbing material. Material from peaks B and C contained very little 260 nm absorbing material (0.27 and 0.24% respectively) while peaks A and D contained substantial amounts.

Fractions B and C reacted strongly with Bratthall group *a* antisera (Table 3). Fractions A and D did not react at all, while Fractions B and C each produced a 4+ reaction in capillary precipitin tests. Examination of acid hydrolysates of these two antigens showed the same major components were present to varying degrees.

Examination of antigenic fractions for purity--Samples of Fractions B and C were placed in an analytical ultracentrifuge in order to determine both approximate molecular size and purity (Fig. 3). A single peak was shown for Fraction B, while Fraction C gave a single diffuse peak due apparently to lower molecular weight. Measurement of the peaks gave an S_{20} value of 2.70 for Fraction B and 1.23 for Fraction C.

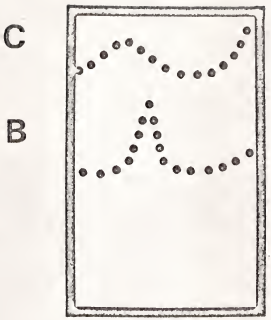
Precipitation of the antigens by specific antibody employing the Ouchterlony double-diffusion technique is shown in Fig. 4. Both Fractions B and C reacted with the group *a* antiserum, but only partially cross-reacted with each other. However, the appearance of single bands indicated a lack of gross contamination by other polymers.

Table 3. Antigenic analysis of pooled material eluted from agarose columns using antiserum prepared against *S. mutans* AHT

Peak*	Fraction numbers	Capillary precipitin reaction
A	55-83	-
B	84-125	4+
C	135-185	4+
D	186-210	-

*In each case, fractions within a peak area were pooled, lyophilized, and samples (1 mg/ml) were prepared in s-EDTA for analysis.

Fig. 3. Analytical ultracentrifugation analysis of purified Fractions B and C derived from *S. mutans* AHT. Samples in saline were centrifuged for 128 min at 59,780 rpm and S_{20} values were calculated according to the method of Schachman (74). Material moved from left to right.



Fraction	Concentration*	S_{20}
B	8.5	2.70
C	9.6	1.23

* mg/ml saline

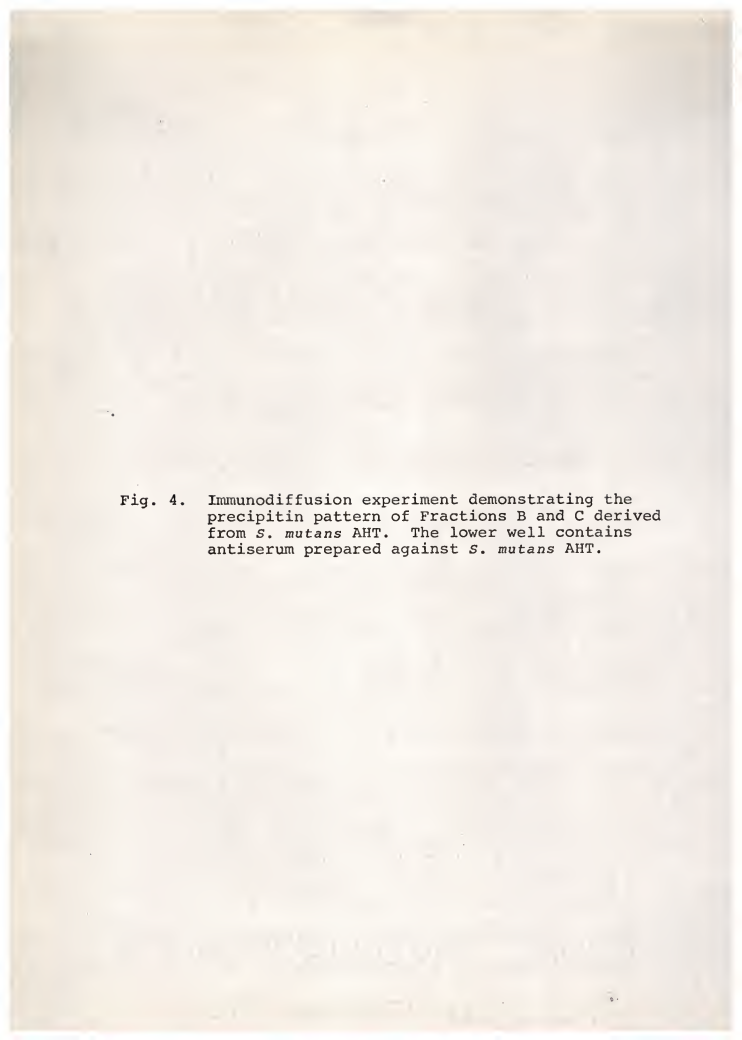
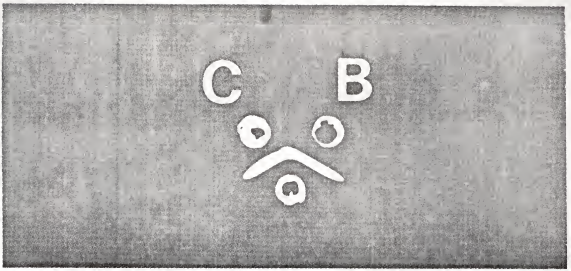


Fig. 4. Immunodiffusion experiment demonstrating the precipitin pattern of Fractions B and C derived from *S. mutans* AHT. The lower well contains antiserum prepared against *S. mutans* AHT.


C
B

Qualitative and quantitative chemical analysis--The chemical characterization of the antigenic fractions is shown in Table 4. Both polymers contained phosphorus, glycerol, galactose, glucose, and alanine as well as fatty acids. The glycerol to phosphorus ratio of Fraction B was 1.97:1.00 which is typical of a phospholipid moiety. Glucose and galactose were present to a high degree while alanine was present only in small quantities. Fraction C had a glycerol to phosphorus ratio of 1.07:1.00 and was associated with glucose, galactose, and alanine; all indicative of a glycerol teichoic acid. Antisera (obtained from Dr. K. Knox) which had been prepared against *Lactobacillus casei* membrane teichoic acid reacted with Fraction C in a capillary precipitin test. This antiserum is known to be specific for glycerol teichoic acid backbones (K. Knox, personal communication). Since these observations indicated the possibility of a membrane-associated glycerol teichoic acid being present and since others have shown such polymers to be useful group- and type-specific antigens, I decided to pursue further studies only on this fraction.

Alkaline hydrolysis and identification of resolved products--Fraction C was base hydrolyzed and its constituent units were separated on a DEAE-cellulose column using a gradient of ammonium carbonate buffer. After initial loading of the hydrolyzed sample onto the column, 200 ml of water were used to elute any non-charged components. Five

Table 4. Chemical characterization of antigenic fractions derived from membranes of *S. mutans* AHT

Compound*	Fraction B			Fraction C		
	µg/mg	µmoles/mg	mole ratio	µg/mg	µmoles/mg	mole ratio
Phosphorus	14.61	0.47	1.00	59.94	1.93	1.00
Glycerol	85.28	0.93	1.97	198.94	2.16	1.07
Galactose	306.57	1.70	3.62	173.86	0.97	0.49
Glucose	130.59	0.73	1.55	140.74	0.78	0.39
Alanine	3.21	0.04	0.08	23.70	0.27	0.13

*Fatty acids were present in both fractions.

phosphorus-containing peaks then were resolved by the buffer gradient (Fig. 5). The water and phosphorus-containing fractions were pooled separately, lyophilized, and brought to volume (0.5 ml) with water for further analysis.

Paper chromatography of material from peaks II, III, and IV demonstrated the presence of glycerol phosphate, phosphorus, and glycerol diphosphate, respectively (Table 5). Each sample was eluted with water from the resolved chromatograms, lyophilized, and treated with alkaline phosphatase. The enzyme-treated products were chromatographed further in solvent system B to show the presence of free glycerol and phosphorus after which each treated sample was assayed directly for glycerol and phosphorus. Controls were run for the presence of glycerol and phosphorus contaminating the enzyme. Fraction II contained phosphorus and glycerol in a ratio of 1.00:0.94 confirming the presence of glycerol phosphate; fraction III only contained phosphorus, while fraction IV contained phosphorus and glycerol in a ratio of 2.08:1.00, confirming the identity of glycerol diphosphate.

Fraction V contained a compound that had the characteristic R_f of diglycerol triphosphate, a compound indicative of a 1-3 phosphodiester linkage in the original polymer (3). The eluted compound was treated with alkaline phosphatase as described above to release the terminal phosphoric

Fig. 5. Separation of alkaline-hydrolyzed Fraction C derived from *S. mutans* AHT on a DEAE-cellulose column. Samples (25 mg) were loaded on the column, followed by partial elution with 200 ml of water. A linear gradient of 0.0-0.2 M ammonium carbonate buffer (pH 8.9) was applied to resolve the charged products. Flow rates of 25-30 ml/hr were maintained and fractions (2.5 ml) were collected. I, II, III, IV, and V signify phosphorus-containing fractions.

Phosphorus (820 nm)
2.0
1.0
I
II
III
IV
V
10
30
50
70
Tube Number
0.2
0.1
$(NH_4)_2CO_3$ (M)

Table 5. Identification of fractions obtained by ion-exchange chromatography of alkaline-hydrolyzed Fraction C derived from *S. mutans* AHT

Fraction[a]	Compound
Water	Glycerol Glycosyl glycerides Fatty acids[b]
I	Fatty acids[b]
II	Glycerol phosphate
III	Phosphorus
IV	Glycerol diphosphate
V	Diglycerol triphosphate

[a]Fractions I and III also contained minor unidentifiable compounds reacting with the silver nitrate reagent.

[b]Not identified

acid groups and then the ratio of total phosphorus to enzyme-labile phosphorus was determined. A ratio of 3.07:2 was obtained. Again controls were run for the presence of phosphorus contaminating the enzyme. This phosphorus ratio plus the R_f of the newly liberated product, diglycerol phosphate, was further evidence for the identity of fraction V as diglycerol triphosphate.

The water fraction was chromatographed in solvent system B. Three distinct spots could be detected with the modified silver nitrate spray reagent. The fastest moving compound co-chromatographed with glycerol and was identified after elution from unsprayed papers as glycerol (Table 6) by specific enzymatic analysis as described in Materials and Methods.

Two slower moving compounds were also detected (R_{gly} 0.544 and 0.214). Both compounds were eluted with water from the resolved chromatograms, lyophilized, and acid hydrolyzed. After lyophilization, each sample was brought up to volume (1.0 ml) with 2 M HCl and hydrolyzed in sealed ampoules for 3 hr at 100 C. The acid was removed by lyophilization and the residue was washed three times with 1.0 ml aliquots of water. The final dried samples were brought up to volume (0.7 ml) with 0.067 M sodium phosphate buffer (pH 7.0) and analyzed for their glycerol, glucose, and galactose contents. Paper chromatography in solvent system B demonstrated these three compounds to be present in each acid hydrolysate. The compound with the

Table 6. Composition of water fraction obtained by ion-exchange chromatography of alkaline-hydrolyzed Fraction C derived from *S. mutans* AHT

$R_{glycerol}$	Molar ratio (gly:glu:gal)*
1.000	-
0.544	1:0.38:0.07
0.214	1:0.93:0.95

*Glycerol, glucose, and galactose

R_{gly} of 0.544 contained glycerol, glucose, and galactose in a molar ratio of 1:0.38:0.07 while the slower compound had a molar ratio of 1:0.93:0.95. The compound with the R_{gly} of 0.214 would appear to be a major glycoside in the native teichoic acid polymer. The identity of the other compound was not further determined. Minor amounts of several unidentifiable compounds were also found in fractions I and III as well as after fraction V.

Polymer chainlength--Table 7 shows an average chain-length of 24 glycerol phosphate units per polymer. Teichoic acid (Fraction C) (1 mg) was treated with alkaline phosphatase (0.5 mg) in 0.5 ml 0.02 M ammonium carbonate buffer (pH 9.5) for 18 hr at 37 C to release terminal phosphoric acid groups (5), shown as enzyme labile. The total phosphorus content was measured and the ratio of total phosphorus/labile phosphorus established. Controls were run for contamination of the enzyme with phosphorus.

Ammonolysis and configuration of released products--Samples of Fraction C were shaken with methanol-aq. 2 M ammonia for 3 hr at room temperature to determine if alanine was linked to the teichoic acid polymer in the characteristic alkaline-labile ester linkage. The samples were chromatographed and free alanine and its amide were separated and identified (Table 8).

Treated samples were chromatographed also in solvent system B and sprayed with either D- or L-amino acid

Table 7. Chainlength of glycerol teichoic acid (Fraction C) derived from *S. mutans* AHT

Phosphorus	µg/ml	µmole/ml	Chainlength (total P/enzyme labile P)
Total	270.31	8.75	24
Enzyme labile	11.25	0.36	

Table 8. Release of ester-linked alanine through ammonolysis of *S. mutans* AHT glycerol teichoic acid (Fraction C)

Compound	R_f*	
	Standard	Unknown
Alanine	0.54	0.54
Alanine amide	0.69	0.69

*n-Propanol:ammonia:water (6:3:1)

oxidase. The released alanine was identified as the D-isomer (Table 9).

Quantitative precipitin reaction--A quantitative precipitin experiment was run in order to demonstrate the equivalence point for hapten inhibition experiments. The results are shown in Fig. 6. Antisera to whole cells prepared as described in Materials and Methods were incubated with varying amounts of teichoic acid (Fraction C) for 4 days at which time the precipitates were collected and analyzed for their antibody protein contents. Controls for non-specific precipitating antibody were run and were negative. All samples were run in triplicate. As can be seen in Fig. 6, equivalence was obtained at approximately 250 µg of teichoic acid. One interesting point is that at equivalence this membrane-associated polymer precipitated 10.5 mg/ml antibody protein, an extremely high titer.

Hapten inhibition--After diluting the antisera 1:10, a quantitative precipitin inhibition assay was set up in duplicate using 75 µmoles of inhibitor in each case. Various components of the antigen or chemical derivatives were tested for possible inhibition of antibody-antigen precipitation (Table 10). As can be seen from the data, the greatest amount of inhibition was obtained by β-galactosides. Galactose also exhibited a strong inhibition which would be expected since it is free to rotate between

Table 9. Alanine configuration in *S. mutans* AHT glycerol teichoic acid (Fraction C)

Compound	Amino acid oxidase	
	L	D
Fraction C	-	+
D-alanine	-	+
L-alanine	+	-

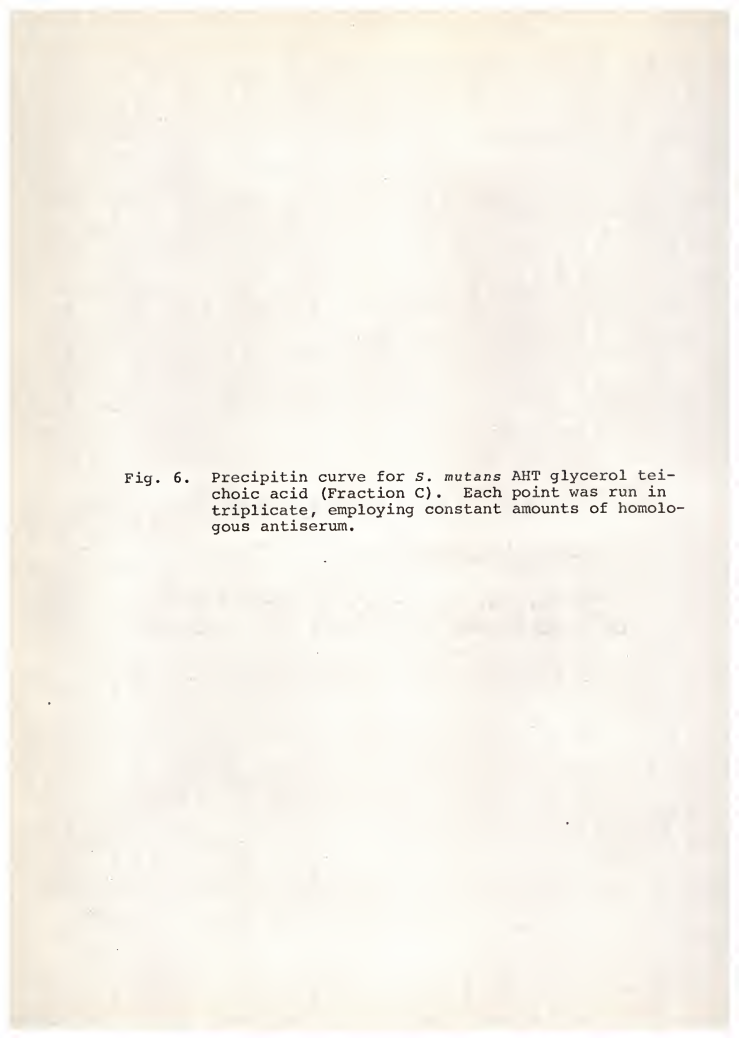


Fig. 6. Precipitin curve for *S. mutans* AHT glycerol teichoic acid (Fraction C). Each point was run in triplicate, employing constant amounts of homologous antiserum.

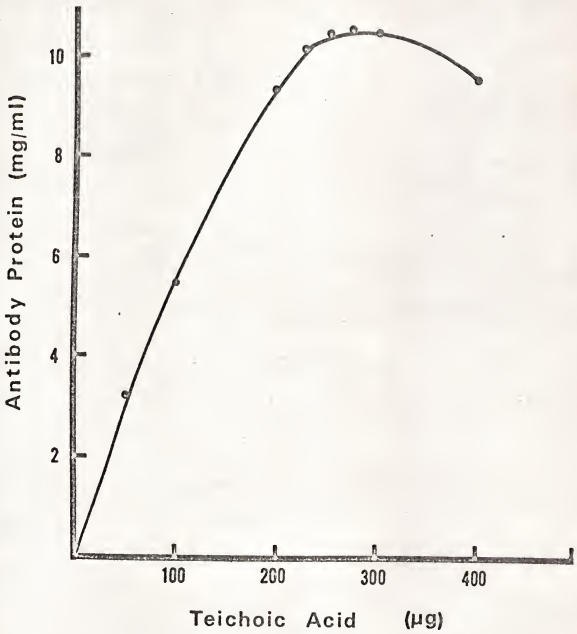

Antibody Protein (mg/ml)
10
8
6
4
2
100
200
300
400
Teichoic Acid (µg)

Table 10. Hapten inhibition of the quantitative precipitin reaction between teichoic acid (Fraction C) and antiserum against *S. mutans* AHT

Inhibitor[a]	Percent inhibition Exp I	Exp II
Galactose	72.3	80.4
α-methyl galactopyranoside	56.3	
β-methyl galactopyranoside	--	86.7
Thiomethyl-β galactopyranoside	88.1	
Glucose	27.5	
α-methyl glucopyranoside	16.8	
β-methyl glucopyranoside	22.2	
Sucrose (glu-fru[b], α1-2)	15.1	20.1
Lactose (gal-glu, β1-4)	75.5	72.7
Glycerol phosphate	14.7	
D-alanine	22.2	
L-alanine	19.6	

[a] 75 μmoles

[b] fructose

its α and β configurations in aqueous solution. Glucose and its α- and β-methyl derivatives inhibited to a much lesser extent. Glycerol phosphate, D- and L-alanine were also tested but only minor amounts of inhibition were noted.

Capillary precipitin and Ouchterlony analysis--Other members of Bratthall's group *a* were surveyed for antigenic cross-reactivity with Fraction C obtained from *S. mutans* AHT. Mild Lancefield extracts were prepared from all organisms previously described in Table 2. All extracts from group *a* organisms demonstrated a 3+ or 4+ reaction with antisera prepared against whole cells of *S. mutans* AHT. Extracts from organisms listed in groups *b* and *c* did not cross-react while extracts from group *d* organisms did cross-react with the antisera. This is in agreement with the work of Bratthall (14). The group *d* cross-reaction was adsorbed out by incubating heat-killed *S. mutans* B13, a group *d* organism, with the *S. mutans* AHT antisera according to the method of Bratthall (14).

Ouchterlony slides were prepared and are demonstrated in Fig. 7. The teichoic acid (Fraction C) shows a reaction of partial identity with Lancefield extracts of each of the organisms presently listed in group *a* as well as the Lancefield extracts of *S. mutans* AHT. This is indicative of the presence of a similar antigen in each of the other related organisms.

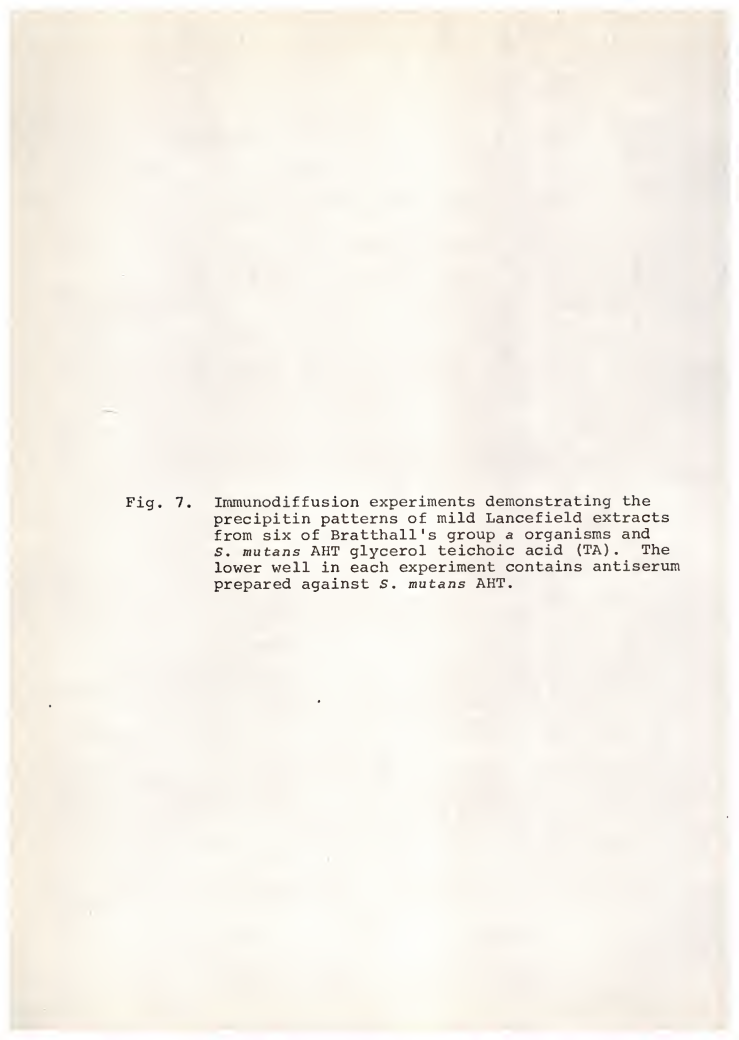
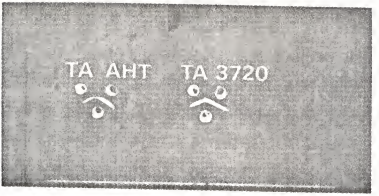
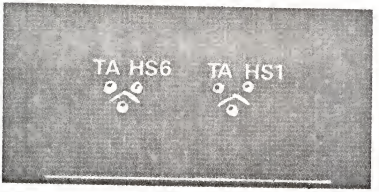


Fig. 7. Immunodiffusion experiments demonstrating the precipitin patterns of mild Lancefield extracts from six of Bratthall's group *a* organisms and *S. mutans* AHT glycerol teichoic acid (TA). The lower well in each experiment contains antiserum prepared against *S. mutans* AHT.


TA AHT
TA 3720

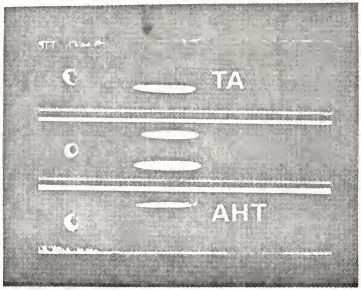

TA HS6
TA HS1

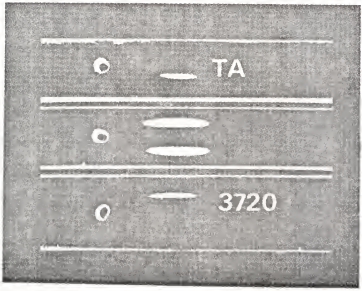

TA E49
TA OMZ61

Immunoelectrophoretic studies--Since the single bands demonstrated by Ouchterlony analysis could, in fact, be multiple antigens with very similar diffusion properties, immunoelectrophoretic analyses were attempted in order to clarify the situation (Figs. 8 and 9). In each preparation the top well contained the pure teichoic acid (Fraction C). The bottom well contained a mild Lancefield extract of the organism tested while the middle well contained an equal mixture of the two. In all preparations, the antigens migrated a similar distance towards the cathode and exhibited only single bands after antibody had been added to the troughs and incubated for 14 hr at 4 C. No additional bands appeared when incubations lasted 7 days at 4 C. The mixed preparations showed identical single bands indicating the antigens were all similar or identical to the *S. mutans* AHT membrane-associated glycerol teichoic acid.

As further evidence to identify the teichoic acid as a group-specific determinant for Bratthall's group *a*, lactose was incorporated into the agar used for immunoelectrophoresis (248 µmoles/ml). After each preparation was electrophoresed and incubated for 18 hr at 4 C with antisera, no precipitin bands had formed. Controls using extracts from group *d* organisms formed characteristic bands during this time period. The omission of lactose allowed characteristic group *a* bands to appear in all cases.

Fig. 8. Immunoelectrophoretic experiments demonstrating the precipitin patterns of mild Lancefield extracts from two of Bratthall's group *a* organisms and *S. mutans* AHT glycerol teichoic acid (TA). The middle well contains an equal mixture of the substances in the outer two wells. The two troughs contain antiserum prepared against *S. mutans* AHT.


TA
AHT


TA
3720

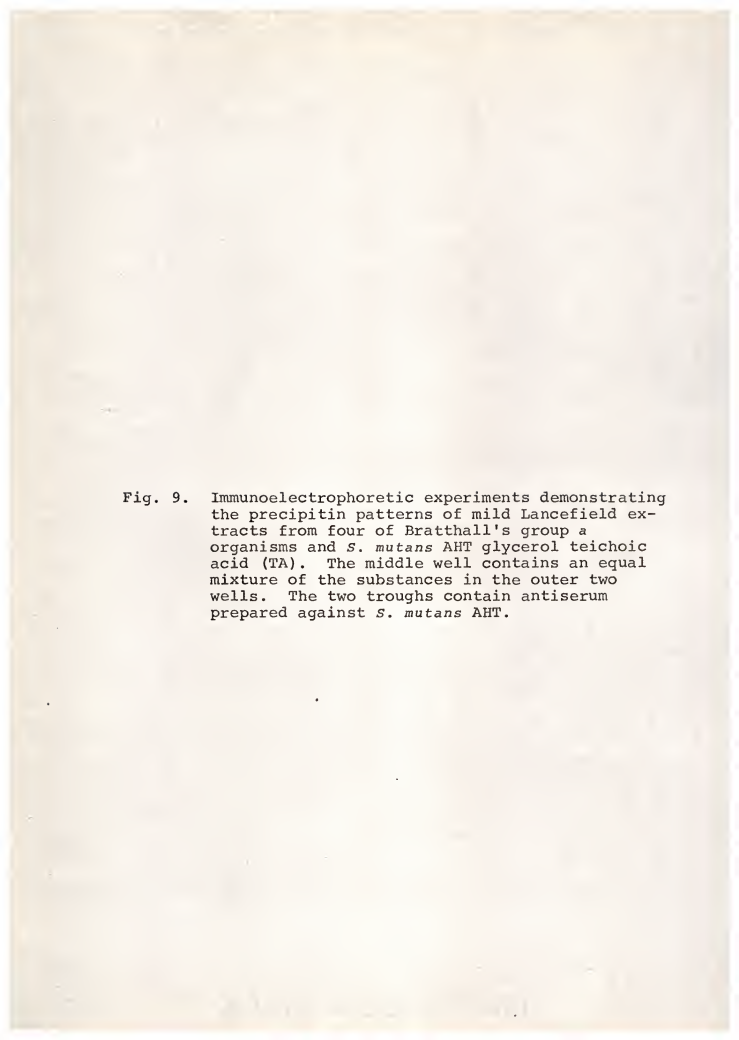
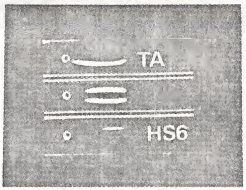
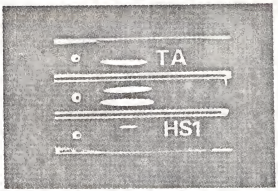
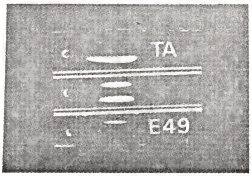
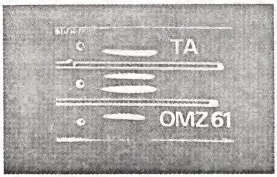


Fig. 9. Immunoelectrophoretic experiments demonstrating the precipitin patterns of mild Lancefield extracts from four of Bratthall's group *a* organisms and *S. mutans* AHT glycerol teichoic acid (TA). The middle well contains an equal mixture of the substances in the outer two wells. The two troughs contain antiserum prepared against *S. mutans* AHT.


TA
HS6


TA
HS1


TA
E49


TA
OMZ61

DISCUSSION

A membrane-associated glycerol teichoic acid antigen has been isolated from *S. mutans* AHT and a similar antigen has been demonstrated to be present in each of the other Bratthall group *a* organisms studied.

TCA-extracted material was resolved into two phosphorus-containing antigenic fractions (B and C) by agarose chromatography (Table 3). Fraction B was shown to contain glycerol and phosphorus in a ratio of 2:1 (Table 4) which is characteristic of a phospholipid moiety. Sugars (glucose and galactose) and fatty acids were also present. Recently Shaw *et al.* (77) and Ambron and Pieringer (1) identified a phospholipid in the membrane of *Mycoplasma laidlawii* and *S. faecalis* as glycerylphosphoryldiglycosyl diglyceride. The new phospholipid contained glycerol and phosphorus in a ratio of 2:1 as well as fatty acids and sugars. This is the only phospholipid presently known with the above glycerol to phosphorus ratio.

Fraction C contained glycerol and phosphorus in a ratio of 1:1 as well as containing glucose, galactose, alanine, and fatty acids (Table 4). Fraction C was identified as a glycerol teichoic acid by its alkaline-hydrolysis products, especially diglycerol triphosphate, the compound

characteristically released from 1-3 phosphodiester-linked glycerol teichoic acids.

Alanine was identified as its alkaline-labile ester-linked D-isomer (Tables 8 and 9). Approximately one out of every ten C-2 hydroxyl groups of glycerol are substituted by this amino acid (Table 4).

Recently Wicken and Knox (88) and Toon *et al.* (83) isolated "lipoteichoic acids" from *Lactobacillus sp.* and *S. faecalis* membranes using a phenol-water extraction procedure. The "lipoteichoic acids" were demonstrated to be glycerol teichoic acids associated with phospholipids and glycolipids. Re-isolation of the membrane-associated teichoic acid from *S. mutans* AHT by the phenol-water extraction procedure should clarify the role of the lipids associated with Fraction C. TCA extraction removes most of the phospholipids associated with glycerol teichoic acids (88).

Even after the teichoic acid polymer was extracted by 10% TCA, a polymer with an average chainlength of 24 repeating units was isolated (Table 7). This value is in agreement with those in the literature for other organisms, but the *in vivo* chainlength possibly is much greater.

Alkaline hydrolysis also was useful in determining the chemical structure of glycosidic side groups (Table 6). It would appear that the glycoside containing equimolar glycerol, glucose, and galactose (R_{gly} 0.214) represents

the major side group associated with the teichoic acid. Hapten inhibition analysis (Table 10) demonstrated that a ß-galactoside could be the antigenic side group. Glucose exhibited inhibition to a much lesser degree. The haptenic side group therefore could be composed of galactose linked by a beta linkage to glucose; or conversely glucose linked to galactose which in turn is linked to the 2-hydroxyl position of glycerol through a beta linkage.

Unfortunately only small amounts of this glycoside would be obtained. Since alkaline hydrolysis continues only when there is a free adjacent hydroxyl group present, the ester-linked disaccharide and D-alanine moieties possibly retarded the complete hydrolysis of the teichoic acid. From the quantitative data in Table 4, it can be determined that approximately six out of every ten C-2 hydroxyl groups of glycerol are substituted with either of the above-mentioned substituents. It is probable that the unidentifiable compounds in fractions I, III, and after fraction V are incomplete alkaline-hydrolysis products such as diglycerol triphosphate which is an unsubstituted derivative.

Comparative immunodiffusion analysis of Lancefield extracts of the six organisms found in Bratthall's group *a* and glycerol teichoic acid derived from *S. mutans* AHT indicates that each of the organisms contains a similar antigen (Fig. 7). The spurs indicating incomplete identity could possibly be due to the presence of other polymers with

the identical or similar hapten. Fraction B also was antigenic and contained both glucose and galactose attached to a phospholipid. Since immunodiffusion analysis demonstrated a spur between Fractions B and C (Fig. 4), it would seem possible that this phospholipid contains a haptenic disaccharide identical or similar to the one associated with the teichoic acid. Matsuno and Slade (64) have shown that the Lancefield group A group-specific hapten N-acetylglucosamine is also found attached as a side group to a membrane-associated glycerol teichoic acid. The originally described group A antigen is a polymer of rhamnose with N-acetylglucosamine acting as the hapten (67). Studies are underway to determine if Fraction B is present in each of the Bratthall group *a* organisms. Since Fractions B and C contain the same components it is possible that either or both fractions could represent the group-specific antigen.

Comparative immunoelectrophoretic analyses (Figs. 8 and 9) of the Lancefield extracts and the glycerol teichoic acid derived from *S. mutans* AHT demonstrated single bands all of which migrated the same distance to the cathode. When lactose was added to the diffusion medium, the appearance of all bands was inhibited indicating a common hapten exists in each group *a* strain of *S. mutans* studied.

Analytical ultracentrifugation, immunodiffusion, and immunoelectrophoresis demonstrated that Fraction C was apparently composed of a single compound. TCA degrades

the backbone of the teichoic acid very slowly under the conditions used for isolation (5). Even though the results of the above techniques suggested the presence of a single compound, there likely is a family of multi-sized polymers present. This would explain why precipitin bands (Figs. 4, 7, 8, and 9) were not as fine as those usually formed with protein antigens.

Wicken and Knox (89) were able to identify four out of the six serologic groups of the lactobacilli upon isolating antigenic teichoic acids. Bleiweis *et al.* (10) have demonstrated the presence of a glycerol teichoic acid in the walls of *S. mutans* BHT, a Bratthall group *b* organism. This fraction reacted with antisera prepared against *S. mutans* BHT and *S. mutans* FA1 but not against *S. mutans* AHT antisera. Conversely, the group *a* glycerol teichoic acid did not react with either of the two group *b* antisera. The data presented in this dissertation indicate that this polymeric type may be useful in immunochemical characterizations of serologic groups of the cariogenic species *S. mutans*. The four other groups of *S. mutans* contain group-specific antigens as demonstrated by Bratthall (14), but these still remain undefined. The work presented in this dissertation is the first immunochemical characterization of a possible group-specific determinant in a cariogenic organism.

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BIOGRAPHICAL SKETCH

Ivo Jaap van de Rijn was born September 30, 1946, in Dordrecht, Netherlands. His family immigrated to the United States in 1952. In June, 1964, he was graduated from Central High School, Philadelphia, Pennsylvania. In June, 1968, he received the degree of Bachelor of Science in premedicine from The Pennsylvania State University. From September, 1968, until the present time, he has pursued work toward the degree Doctor of Philosophy in the Department of Microbiology, University of Florida.

Ivo Jaap van de Rijn is married to the former Arlene Fay Safer.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Arnold S. Bleiweis, Chairman
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Henry C. Aldrich
Associate Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward M. Hoffmann
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1972

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